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Abbreviations

ATNC, apparent total N-nitroso compounds; CRC, colorectal cancer; DM, dry matter; GSH-Px, glutathione peroxidase; HEX, hexanal; MDA, malondialdehyde; NOC, N-nitroso compounds; O^6 MeG, O^6-methyl-2-deoxyguanosine; OPLS-DA, orthogonal projections to latent structures discriminant analysis; PCA, principal component analysis; PCC, protein carbonyl compounds; RSNO, nitrosothiols; FeNO, nitrosyl iron compounds; SCFA, short chain fatty acid; TSP, 3-(trimethylsilyl)-propionate; 4-HNE, 4-hydroxynonenal.

Abstract

Intake of red and processed meat has been suspected to increase colorectal cancer risk potentially via endogenous formation of carcinogenic *N*-nitroso compounds or increased lipid- and protein oxidation. Here we investigated the effect of inulin fortification of a pork sausage on these parameters. During four weeks, healthy Sprague-Dawley rats (n = 30) were fed one of three diets; inulin-fortified pork sausage, control pork sausage or a standard chow diet. Fecal content of apparent total N-nitroso compounds (ATNC), nitrosothiols and nitrosyl iron compounds (FeNO) were analyzed in addition to liver metabolism and oxidation products formed in liver, plasma and diets. Intriguingly, inulin fortification reduced fecal ATNC ($p = 0.03$) and FeNO ($p = 0.04$) concentration. The study revealed that inulin fortification of processed meat could be a strategy to reduce nitroso compounds formed endogenously after consumption.

Key words Fiber-fortification, inulin, processed meat, nitroso compounds, oxidation

1. Introduction

Consumption of red and particularly processed meat has been associated with a possible increased risk of colorectal cancer (CRC) (Chan et al., 2011). Among the major hypotheses explaining this possible association, endogenous formation of *N*-nitroso compounds (NOCs) following red or processed meat ingestion has been suggested (Hughes, Cross, Pollock and Bingham, 2001). Many NOCs are suspected to be carcinogenic and red meat consumption has been shown to dose-dependently increase the fecal excretion of NOCs (Hughes et al., 2001). NOCs can lead to alkylation of DNA, resulting in the formation of pro-mutagenic DNA adducts. This can induce G:C→ A:T mutations, which might eventually initiate carcinogenesis (Gottschalg, Scott, Burns and Shuker, 2007, Mirvish, 1995). Intake of red meat has in fact been shown to increase DNA adduct formation in mice and human, including the pro-mutagenic DNA adduct O^6-methyl-2-deoxyguanosine (O^6 Meg) (Le Leu et al., 2015, Winter et al., 2011). Heme iron, a component of red and processed meat, has been suggested to stimulate NOC formation following red or processed meat ingestion (Cross, Pollock and Bingham, 2003). Endogenous NOCs are likely formed via various routes throughout the gastrointestinal tract, including acid and bacterial catalyzed reactions, generally as a result of the reaction between nitrosating agents and nitrosable substrates (Hughes, Magee and Bingham, 2000). Acid catalyzed nitroso compound formation occurs mainly in the stomach, where nitrosating agents, such as dietary nitrite reaching the stomach, result in the formation of various nitroso compounds (Kobayashi, 2018). The acidic environment of the stomach has been found to favor the formation of nitrosothiols, which has been suggested to be the initial step in the formation of nitroso compounds in the gastrointestinal tract (Kuhnle et al., 2007). The nitrosothiols formed in the stomach might be precursors for NOCs formed further down the GI tract, as the increasing pH favors their release of NO (Kuhnle and Bingham, 2007).

In the small intestine, it has been suggested that heme might be nitrosylated by nitrite or the NO released from nitrosothiols, making it possible for the nitrosylated heme to act as a nitrosating agent increasing the formation of NOC (Kuhnle et al., 2007).

Bacterial catalyzed NOC formation has been found to require the presence of bacteria with nitrite and nitrate reductase enzymes activity (Calmels, Ohshima and Bartsch, 1988, Calmels, Ohshima, Henry and Bartsch, 1996). Intriguingly, a study investigating the formation of NOCs in germ free rats found that the presence of a colonic flora was necessary for NOC formation to occur (Massey, Key, Mallett and Rowland, 1988). In the large intestine, nitrosable substrates formed via protein degradation as well as nitrosating agents are available, providing a site for bacterial catalyzed NOC formation (Hughes et al., 2000).

Besides the suggested role of heme in NOC formation, heme iron in red meat has been suggested to stimulate lipid and protein oxidation (Bastide, Pierre and Corpet, 2011, Van Hecke, Vanden Bussche, Vanhaecke, Vossen, Van Camp and De Smet, 2014). In particular, lipid oxidation may result in the formation of potentially toxic end-products including malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) formed via peroxidation particularly of polyunsaturated fatty acids. Both MDA and 4-HNE have been found to be able to react with DNA to form DNA adducts, whereas 4-HNE also has shown cytotoxic effects (Bastide et al., 2011, Nair, Bartsch and Nair, 2007).

Previous investigations have indicated that dietary fiber consumption has a protective effect against CRC development (Bingham et al., 2003). In fact, earlier studies found that dietary fibers consumed in combination with red meat attenuated meat-induced DNA damage and potential harmful protein fermentation products, whereas fecal short chain fatty acids (SCFAs) concentrations were increased (Le Leu et al., 2015, Toden, Bird, Topping and Conlon, 2007, Winter et al., 2011). Moreover, fiber addition to meat products has shown a lowering effect on lipid oxidation following *in vitro* digestion

(Hur, Lee and Lee, 2014). Thus, we recently demonstrated that inulin fortification of a pork sausage product increased fecal content of SCFAs when fed to healthy rats during a 4-week intervention (Thogersen et al., 2018). Based on the same experimental study, we here investigated whether the incorporation of inulin into a pork sausage product also had a protective effect on the formation of apparent total N-nitroso compounds (ATNC) upon consumption. Compounds-specific denitrosation prior to analysis was used in order to investigate the types of nitroso compounds formed. The effect of inulin fortification on protein and lipid oxidation was examined by measuring oxidation markers in plasma and liver. Furthermore, as the liver is a key metabolic organ, possible hepatic metabolic changes were studied using ^{1}H nuclear magnetic resonance spectroscopy.

2. Materials and methods

2.1 Sausage diets

Two different sausage batches were manufactured for the study, and Table S1 shows the nutritional composition of the experimental diets. The sausages were made from a sausage emulsion of pork meat, pork backfat, which was prepared with a bowl cutter using a standard procedure for frankfurter sausages. After mincing of pork meat and backfat, the remaining ingredients provided in Supporting Information, Table S2, were added to the minced meat. For the inulin-enriched sausages, inulin was added to a fiber content approximating the content in the chow diet (5.6 % compared to 6.05 %). The inulin used was Orafti® HP (Beneo-Orafti, Oreye, Belgium), a long-chain chicory inulin product containing 99.5 % inulin with an average degree of polymerization of 23 ranging from 5 to 60. Inulin fibers were added in their dry form without any pre-treatment. Casings (22/24 lamb casings) were filled with 82 g meat batter to reach a final weight of 75 g after heat treatment. After heat treatment the sausages were frozen at stored at -18 °C until further use.

2.2. Rat intervention and sample collection

Thirty healthy Sprague-Dawley rats (NTac:SD) at the age of four weeks (Taconic, Ll. Skensved, Denmark) were used in this study. The rats were housed in our Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited facility, and randomly housed into ten U1400 cages (Tecniplast, Buguggiate, Italy) on aspen bedding and with enrichment (Tapyei, Harjumaa, Estonia) in groups of three rats per cage following weighing and earmarking. The facility was health monitored according to FELASA guidelines (2014) revealing none of the infections listed. The rats were allowed a seven-day adaptation period during which they were fed a standard chow diet *ad libitum* (Altromin 1324, Brogaarden, Denmark) with free access of water. After adaptation, the rats were randomly divided into three groups receiving one of the following diets during an experimental period of four weeks; 1) Pork sausages enriched with 5.6 % chicory inulin (n = 12), 2) Pork sausages without enrichment (n = 12), 3) standard chow diet (Altromin 1324), (n = 6). Body weight, food and water intake have been published elsewhere (Thogersen et al., 2018).

After the intervention period, fecal samples were collected and the rats were sacrificed according to previously described procedures (Thogersen et al., 2018). After anesthesia by hypnorm/midazolam (diluted 1:1 with sterile water prior to mixing; 0.2 mL/g body weight), heart blood was collected followed by decapitation.. Liver samples were collected by carefully removing the liver. Samples of approximately 2x2 cm were subsequently transferred to Cryotubes and snap frozen in liquid nitrogen. Samples were stored at -80 °C until analysis.

The study was in accordance with Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes and the Danish Animal Experimentation Act (LBK 474 15/05/2014). Specific approval was granted by the Animal Experiments Inspectorate under the Ministry of Environment and Food in Denmark (License No 2012-15-2934-00256).

2.3 Nitroso compounds

Prior to analysis, fecal samples were disrupted using a TissueLyser LT (Qiagen). Approximately 200 mg fecal sample, 500 µL 1.0 mm glass beads (Sigma-Aldrich, St. Louis, MO, USA) and 1 mL HPLC grade water per 200 mg feces were added a 2 mL Eppendorf Tube and sample disruption was conducted for 10 min oscillating at 50 1/s. The samples were centrifuged at 14,000 x g for 15 min at 4 °C and the collected supernatant was stored at -80 °C until further analysis. Nitroso compound determination was based on a previously described method (Kuhnle et al., 2007) with modifications using chemiluminescence detection with an Ecomedics CLD 88 Exhalyzer (Ecomedics, Dürnten, Switzerland). A purge vessel containing 15 mL of a tri-iodide solution (2 g potassium iodide, 1.3 g iodine 40 mL water and 140 mL glacial acetic acid) and heated to 60 °C was connected via a condenser to a wash bottle containing 1 M NaOH. The wash bottle was connected to the Ecomedics CLD 88 Exhalyzer via a polypropylene filter (0.2 µm, Whatman, USA). The NaOH wash bottle and condenser were kept at 0 °C. For mixing injected sample and transferring released NO to the analyzer, nitrogen gas was bubbled through the system and the signal obtained was processed using instrument software Chart v5.5.8 (eDAQ, Australia). Quantification was based on the injection of sodium nitrite (Sigma-Aldrich, Steinheim, Germany) in a concentration range of 1.22-19.5 µM. For the determination of ATNC, 100 µL fecal supernatant were combined with 100 µL 0.1 M N-ethylmaleimide (NEM) and 0.01 M diethylene triamine pentaacetic acid (DTPA) in water to chelate metal iron and preserve nitroso thiols, and 500 µL sulfamic acid solution (50 g/L in 1 M HCl, Fisher Scientific, Loughborough, UK) to remove nitrite, vortex mixed and incubated for 2 min. Subsequently, the solution was injected into the reaction vessel. Nitrosothiol (RSNO) determination was conducted using the procedure prior to injection for ATNC determination followed by the addition of 100 µL aqueous $HgCl_2$ (10 mM). After vortex mixing and 2 min of incubation, the solution was injected into the purge vessel. Likewise, nitrosyl iron compound (FeNO) determination was conducted using the procedure prior to injection for nitrosothiol determination followed by the addition of 100 µL $K_3Fe(CN)_6$ (10 mM). After vortex mixing and 2 min of incubation, the solution was injected into the purge vessel. The difference between mercury(II) stable and unstable compounds was used as a measure of nitrosothiols and the difference between ferricyanide stable and unstable compounds as a measure of nitrosyl iron (Kuhnle et al., 2007).

A possible protective effect of inulin against NOC formation was investigated under *in vitro* acidic conditions. Bovine hemoglobin (Sigma Aldrich, St Louis, USA), hydrochloric acid and chicory inulin (Beneo GmbH, Mannheim, Germany) were mixed resulting in final concentrations of 100 μM, 7 mM and 740 μM for the three constituents, respectively. Sodium nitrite (Sigma-Aldrich, Steinheim, Germany) was added in a final concentration range of 2.5-50 μM for initiation of the reaction. Following incubation for 15 minutes, ATCN determination in 100 μL was conducted using the procedure described above. Incubation of corresponding solutions without addition of inulin was used as control.

2.4 ^{1}H nuclear magnetic resonance spectroscopy (NMR spectroscopy)

Intact liver tissue was analyzed by ^{1}H NMR spectroscopy using high-resolution-magic-angle spinning (HR-MAS) analysis. Approximately 10 mg of liver sample was added to 30 μL HRMAS disposable inserts (Bruker BioSpin, Gmbh, Rheinstetten, Germany) containing 10 μL D_2O with 0.05 % 3-(trimethylsilyl)-propionate (TSP) and subsequently kept at -80 °C until analysis. ^{1}H NMR spectroscopy was conducted using a Bruker Avance III 600 MHz spectrometer operating at a ^{1}H frequency of 600.13 MHz equipped with an HR-MAS probe (Bruker BioSpin, Rheinstetten, Germany). A one-dimensional (1D) Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence with pre-saturation to suppress the water resonance was used. The following parameters were used: number of scans (NS) = 128, spectral width

(SW) = 10417 Hz, data points (TD) = 32768, target temperature = 278 K and a spinning speed of 4200 Hz. Prior to Fourier transformation, the free inductions decays (FIDs) were multiplied by a line-broadening function of 0.3 Hz. The obtained spectra were baseline- and phase corrected and referenced to TSP (0.0 ppm) using Topspin 3.0 (Bruker BioSpin). Data were loaded into MATLAB R2017b (Mathworks Inc., Natick, USA) and spectral regions above 9.8 ppm, below 0.5 ppm and the water signal region (4.9-5.15 ppm) were removed. Spectra were normalized to total area of the spectrum and subdivided into regions of 0.01 ppm. Chenomx NMR Suite 8.13 (Chenomx Inc., Edmonton, Canada) and literature (Beckonert et al., 2010) was used for metabolite assignment. The following multivariate data analysis was conducted using SIMCA 15.0 (Sartorius Stedim Data Analytics AB, Umeå, Sweden). Data were pareto-scaled and principal component analysis (PCA) was conducted followed by construction of an orthogonal projections to latent structures discriminant analysis (OPLS-DA) model using cross validation with seven segments. An OPLS-DA S-line plot was created in order to reveal metabolites important for the separation between dietary treatment groups.

2.5 In vitro digestion of experimental diets

The *in vitro* digestions were performed in triplicate according to a previously described protocol, specific for studying oxidation processes during passage in the gastrointestinal system (Van Hecke et al., 2014). In brief, 4.5 g of the experimental diets were sequentially incubated at 37°C for 5 minutes with 6 mL saliva, 2 hours with 12 mL gastric juice, and 2 hours with 2 mL bicarbonate buffer (1 M, pH 8.0), 12 mL duodenal juice and 6 mL bile juice. After completion, samples were homogenized with an ultraturrax (9500 rpm) and aliquots were stored at -80°C until analysis of lipid and protein oxidation markers.

2.6 Lipid- and protein oxidation

Oxidation parameters were measured in liver, plasma, experimental diets and *in vitro* digests of the diets. Liver extracts were prepared by homogenizing 1 g of liver tissue in 10 mL 1 % Triton-X-100 phosphate buffer (pH 7; 50 mM) for 45 seconds using an ultraturrax homogenizer, followed by centrifugation (15 min, 15,000 g, 4 °C), after which the supernatant was filtered through glass wool. Supernatants were immediately analyzed for malondialdehyde (MDA) and activity of glutathione peroxidase (GSH-Px). The measurement of total (unbound and bound) MDA was based on a previously described method (Van Hecke, Ho, Goethals and De Smet, 2017) with few modifications and was based on the formation of TBARS from the reaction of MDA with 2-thiobarbituric acid (TBA). The absorbance at 532 nm was measured following 1-butanol extraction, and a 1,1,3,3-tetramethoxypropane standard curve was used for quantification of MDA. The activity of GSH-Px in plasma and liver extracts was determined by measuring the oxidation of NADPH whereby one unit of GSH-Px activity was defined as the amount of extract needed to oxidize 1 µmol of NADPH per min at 25 °C (Hernández, Zomeño, Ariño and Blasco, 2004). Measurement of protein carbonyl compounds (PCC) was based on a previously described method (Ganhão, Morcuende and Estévez, 2010) and based on the formation of a stable 2,4 dinitrophenylhydrazone product as a result of carbonyl groups reacting with 2,4-dinitrophenylhydrazine (2,4-DNPH). Unbound reactive 4-HNE and HEX were measured in diets and *in vitro* digests by HPLC following their derivatization with cyclohexanedione as previously described (Van Hecke et al., 2017).

2.7 Statistical analysis

Values are given as mean ± SEM. For determination of statistical differences between mean values of the three dietary treatment groups, one-way ANOVA were performed followed by Tukey's honest significant different (HSD) test when significant differences were found. For comparison of nitroso compound formation between the two sausage-based diet groups as well as ATNC formation under acidic conditions, two-sample t-test was conducted. For the two sausage-based diet groups, nitroso compound formation below the detection limit was set to zero. P-values < 0.05 were considered significant. Pearson correlations with Bonferroni-Holm corrections and p-value < 0.05 were calculated to investigate possible correlations between relative abundance of fecal bacteria and NOC concentrations. Statistical analyses were conducted using MATLAB R2017b (Mathworks Inc., Natick, USA).

3. Results

In the present study, 30 healthy rats were fed three different diets; inulin-fortified pork sausage product, control pork sausage product or a standard chow diet, during an intervention period of four weeks. We have formerly reported metabolomics analyses of fecal and blood samples collected from the rats (Thogersen et al., 2018). Here nitroso compounds excretion, the liver metabolome and lipid and protein oxidation markers were measured to examine a possible beneficial effect of inulin-fortification on these parameters. Body weight did not differ between dietary treatment groups by the end of the study (Thogersen et al., 2018).

3.1 Nitroso compounds

For all of the measured nitroso compounds, i.e. ATNC, nitroso thiols and nitrosyl haem, the fecal concentration was below the detection limit following chow diet consumption. In general, the highest concentrations were observed upon consumption of the control sausage. The concentration of ATNC was found to be significantly reduced after consumption of inulin-fortified sausage (1.39 ± 0.15 µM) compared to the control sausage diet (2.13 ± 0.28 µM) ($p = 0.03$) (Figure 1). Selective denitrosation prior to analysis revealed no significant difference in RSNO ($p = 0.11$) or other unspecified nitroso compounds ($p = 0.29$) after consumption of the two sausage-based diets. A significant reduction in FeNO ($p = 0.04$) was found after consumption of the inulin-fortified sausage (0.79 ± 0.06 µM) compared to control (1.29 ± 0.22 µM). Concentrations for each individual rat can be found in supplementary material (Table S3). A complementary experiment with incubation of bovine hemoglobin and sodium nitrite under acidic conditions with or with the addition of inulin showed no effect of inulin on ATCN formation (Table 1). Calculations of Pearson correlation coefficients with Bonferroni-Holm correction and significance level $p < 0.05$ found no positive correlations between relative abundance of gut bacteria (published elsewhere (Thogersen et al., 2018)) and concentration of nitroso compounds (data not shown).

3.2 Lipid- and protein oxidation

Oxidation analyses of the experimental diets revealed that the chow diet contained higher concentrations of MDA, 4-HNE and HEX compared to the two sausage-based diets, and protein oxidation was increasing in the order control sausages, inulin-enriched sausages and chow diet (Table 2). Analysis of *in vitro* digests of experimental diets revealed higher MDA, 4-HNE, HEX and PCC concentrations in *in vitro* digests of the chow diet compared to the two sausages-based diets. Analyses of the anti-oxidative enzyme system of rat samples revealed higher GSH-Px activity in liver samples from rats fed the two sausage-based diets compared to chow diet ($p < 0.001$), whereas a near-significant ($p = 0.069$) increased GSH-Px activity was observed in plasma samples from rats fed the sausage-based diets compared to standard chow diet (Table 2).

3.3 Liver metabolome

PCA scores plot of spectral data obtained from HRMAS analysis of liver tissue revealed a clear grouping of the rats receiving the standard chow diet in the first component explaining 74.3 % of the variation (Figure 2). No clear separation between the two sausage-based diets could be observed. An

OPLS-DA model comparing rats fed the standard chow diet and rats fed the two sausage-based diets was constructed ($Q^2 = 0.79$) (Figure S1, Supplementary material) and S-line plot revealed that glucose and lipids were among the main drivers of the separation (Figure 3). Chow diet consumption was characterized by higher hepatic glucose levels, whereas consumption of the sausage-based diets was characterized by higher lipid levels in the liver (Figure 3). In addition, a peak at 3.26 ppm appeared important for the separation between the chow diet group and the rats fed the sausage-based diets. The 3.26 ppm peak is most likely arising from betaine and has its highest intensity in the chow diet group. Multivariate data analysis did not show any separation between the two sausage-based diets.

4. Discussion

4.1 Inulin fortification reduces fecal nitroso compound excretion

Red and processed meat intake has been suspected to cause harmful effects on colon health (Chan et al., 2011), whereas dietary fiber consumption has been associated with colonic health benefits (Bingham et al., 2003). Therefore, the inclusion of dietary fibers into processed meat products might be a strategic tool in reducing the potential meat-associated harmful effects on colon homeostasis. We have previously shown that inulin fortification of a pork sausage product positively affected the metabolome and gut microbiota of healthy rats by increasing the fecal concentration of SCFAs as well as the relative abundance of *Bifidobacteria* compared to a corresponding non-enriched sausage (Thogersen et al., 2018). Here we examined the effect of the same inulin fortification of a pork sausage product on the formation of nitroso compounds, the liver metabolome as well as markers of lipid and protein oxidation using a rat model.

Intriguingly, our study demonstrated that inulin fortification reduced the fecal concentration of ATNC compared to the consumption of control sausages without fortification. Compound-specific denitrosation indicated that this was partly ascribed to a reduction in nitrosyl iron compounds. Based on *in vitro* studies, it has previously been proposed that fermentation of non-digestible carbohydrates could lead to a reduced availability of NOC precursors in the form of amines (Allison and Macfarlane, 1989, Silvester, Bingham, Pollock, Cummings and O'Neill, 1997). In addition, under simulated gastric conditions, wheat bran has been demonstrated to act as a nitrite scavenger (Møller, Dahl and Bøckman, 1988). However, human studies investigating the effect of consuming resistant starch or wheat bran in combination with red meat showed no effect on fecal NOC excretion (Bingham et al., 1996, Silvester et al., 1997). NOC can be formed from the reaction of nitrosating agents and nitrosable substrates such as amines formed via fermentation of protein residues reaching the colon (Kobayashi, 2018). This reaction can be catalyzed by colonic bacteria with nitrate- or nitrite reductase enzyme activity (Calmels et al., 1988, Calmels et al., 1996). Hence, the reducing effect that inulin fortification exerts on fecal nitroso compound excretion may be ascribed to a reduction in substrate availability or changes in catalysis of the reaction. Increasing the availability of fermentable carbohydrates in the colon upon high red meat intake might attenuate the formation of protein fermentation products by switching the bacterial fermentation of proteins towards carbohydrate fermentation (Toden et al., 2007, Winter et al., 2011), thereby reducing the availability of substrates for nitroso compound formation. The reducing effect of inulin on ATNC formation might be a result of a high colonic fermentability of inulin compared to other fermentable carbohydrates previously examined in human studies (Bingham et al., 1996, Silvester et al., 1997). We previously demonstrated a strong effect of diet on the gut microbial composition of the rats included in the present study (Thogersen et al., 2018). Hence, the reduced ATNC excretion observed after inulin-fortified sausage consumption may also be associated with changes in abundance of colonic bacteria with nitrate- or nitrite reducing activity. Alternatively, it may be caused by a reduced nitrate reductase activity, as earlier studies have shown a reducing effect of wheat bran and cellulose on this enzyme activity (Mallett, Rowland and Bearne, 1986, Mallett, Wise and Rowland, 1983). The fact that no suppressing effect of inulin on ATNC formation was found after incubation of bovine hemoglobin under *in vitro* acidic conditions suggests that the presence of inulin did not affect an acid-catalyzed ATNC formation expected to take place in the stomach. Thus, the reducing effect of inulin on ATNC formation appears to result from mechanisms taking place further down the gastrointestinal system.

In the literature, the carcinogenicity of the different types of nitroso compounds has been discussed. Hogg 2007 argued that S-nitrosothiols and nitrosyl iron species, in contrast to N-nitroso species, are not tumorogenic and even suggested a possible protective effect of S-nitrosothiol and nitrosyl iron formation, reducing the formation of DNA adduct alkylating agents and increasing excretion (Hogg, 2007). However, others argue that both nitrosothiols and nitrosyl heme may promote the formation of nitroso-compound-specific DNA-adducts (Kuhnle et al., 2007) and *in vitro* studies have shown the ability of nitrosyl heme and nitrosothiols to act as a nitrosating agents (Alkaabi, Williams, Bonnett and Ooi, 1982, Bonnett, Charalambides, Martin, Sales and Fitzsimmons, 1975).

Fecal concentration of all of the measured nitroso compounds for rats fed the standard chow diet were below the detection limit. The lower fecal concentration of nitroso compounds after chow diet consumption compared to the two sausage-based diets is in accordance with earlier findings showing lower fecal concentrations of nitroso compounds after consumption of a vegetarian diet compared to high red meat diet (Kuhnle et al., 2007). This is likely caused by an expected higher heme iron content in the sausage based diets compared to the chow diet (Cross et al., 2003) and the addition of sodium nitrite salt to the sausages.

4.2 Effect of diet on liver metabolome and oxidation products

Liver metabolism is crucial to the organism, making it a key metabolic organ. HRMAS analysis of intact liver tissue revealed a clear separation of the chow diet group from the two sausage-based diet groups when multivariate data analysis of ^{1}H NMR spectral data was conducted. Liver tissue from rats fed the sausage-based diets was characterized by higher amounts of lipids, whereas the chow diet group was characterized by higher hepatic glucose levels. This finding can likely be ascribed to the higher dietary fat and carbohydrate intake for the sausage-based diet groups and chow diet group, respectively. The results are consistent with earlier findings that the liver metabolome is influenced by metabolic status and can be modified by diet, revealing increased hepatic glucose and lipid content after consumption of high-carbohydrate or high-fat diet, respectively (Bertram, Larsen, Chen and Jeppesen, 2012).

No effect of inulin fortification of the pork sausage product was found on lipid and protein oxidation end products in plasma or liver samples nor in *in vitro* digests of experimental diets. Previous studies found a lowering effect of fiber addition to meat products on lipid oxidation after *in vitro* digestion, probably explained by a lowering effect of fibers on lipid digestion (Hur et al., 2014, Hur, Lim, Park and Joo, 2009). In addition, Toden et al., 2010 found a reducing effect of high amylose maize starch (HAMS) on plasma MDA concentrations in plasma samples of rats fed chicken or beef with or without HAMS (Toden, Belobrajdic, Bird, Topping and Conlon, 2010). Differences in physicochemical properties characterizing different dietary fibers might affect the ability of a specific fiber to reduce lipid digestion in meats in addition to differences in lipid content and lipid size of the meat as suggested by Hur et al. 2009 (Hur et al., 2009).

Intriguingly, oxidation analysis of the experimental diets and *in vitro* digests of diets revealed higher concentrations of oxidation products in the chow diets compared to the two sausage-based diets. It has previously been shown that nitrite curing of pork meat reduced the formation of oxidation products compared to corresponding uncured meat after *in vitro* digestion (Van Hecke et al., 2014), which could explain why the two sausage-based diets show lower oxidation compared to the chow diet. The higher oxidation products in chow diet and *in vitro* digests of the chow diet could also be caused by a higher content of reducing sugars in the chow diet, since reducing sugars might to be able to accelerate oxidation (Yamauchi, Goto, Kato and Ueno, 1984). The dry characteristics of the chow diet as well as a longer storage time and higher storage temperature of the chow diet compared to the sausage-based diet might also contribute to the observed increased oxidation (Lin, Hsieh and Huff, 1998).

The analysis of oxidation products of experimental diets and *in vitro* digests of diets were conducted on equal amounts of fresh matter. However, the dry matter content of the chow diet is lower than that of the sausage-based diets being 89 %, 43 % and 42 % for chow diet, inulin-enriched sausage and control sausage, respectively. Therefore, lipid oxidation products per gram dry matter of experimental diets were calculated and are given in supplementary material, Table S4. According to the calculations, 4-HNE and HEX were still significantly higher in the chow diet, but for MDA, the calculations showed the highest concentration in the inulin-fortified sausages.

The higher degree of oxidation in the chow diet compared to the sausage-based diets prior to ingestion was not reflected in plasma or liver samples of the rats, where no differences in oxidation products between diets groups were found. Intriguingly, despite the chow diet being more oxidized prior to ingestion, an increased GSH-Px activity was observed in liver as well as a near-significant increase in plasma from rats fed the sausage-based diets compared to the chow diet. A high GSH-Px activity can be an indication of a higher level of oxidative stress, since GSH-Px reduces lipid hydroperoxides formed via oxidation of unsaturated fatty acids, thereby functioning as a defense mechanism against the formation of toxic oxidation end-products (Bastide et al., 2011). Thus, the higher fat content, including polyunsaturated fatty acids, in the sausage-based diets could potentially give a higher oxidative stress during digestion compared to that of the chow diet resulting in increased GSH-Px activity.

In conclusion, inulin fortification of a pork sausage product reduced fecal content of ATNC and FeNO compared to a non-enriched sausage in healthy rats, indicating a protective effect of inulin against nitroso compound formation. Although no effect of fiber fortification was found on oxidation products, our results indicate a potential of using inulin fortification of processed meat products as an approach to reduce the formation of potentially carcinogenic nitroso compounds.

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Conflict of interest

The authors declare no conflict of interest.

References

Alkaabi S. S., Williams D. L. H., Bonnett R., Ooi S. L. A KINETIC INVESTIGATION OF THE THIONITRITE FROM (+/-)-2-ACETYLAMINO-2-CARBOXY-1,1-DIMETHYLETHANETHIOL AS A POSSIBLE NITROSATING AGENT. *J Chem Soc-Perkin Trans 2*. **1982**(2):227-30.

Allison C., Macfarlane G. T. Influence of pH, nutrient availability, and growth rate on amine production by Bacteroides fragilis and Clostridium perfringens. *Applied and Environmental Microbiology*. **1989**;55(11):2894-8.

Bastide N. M., Pierre F. H. F., Corpet D. E. Heme Iron from Meat and Risk of Colorectal Cancer: A Meta-analysis and a Review of the Mechanisms Involved. *Cancer Prev Res*. **2011**;4(2):177-84.

Beckonert O., Coen M., Keun H. C., Wang Y., Ebbels T. M., Holmes E., Lindon J. C., Nicholson J. K. High-resolution magic-angle-spinning NMR spectroscopy for metabolic profiling of intact tissues. *Nature protocols*. **2010**;5(6):1019-32.

Bertram H. C., Larsen L. B., Chen X., Jeppesen P. B. Impact of high-fat and high-carbohydrate diets on liver metabolism studied in a rat model with a systems biology approach. *Journal of agricultural and food chemistry*. **2012**;60(2):676-84.

Bingham S. A., Day N. E., Luben R., Ferrari P., Slimani N., Norat T., Clavel-Chapelon F., Kesse E., Nieters A., Boeing H., Tjonneland A., Overvad K., Martinez C., Dorronsoro M., Gonzalez C. A., Key T. J., Trichopoulou A., Naska A., Vineis P., Tumino R., Krogh V., Bueno-de-Mesquita H. B., Peeters P. H., Berglund G., Hallmans G., Lund E., Skeie G., Kaaks R., Riboli E. Dietary fibre in food and protection against colorectal cancer in the European Prospective Investigation into Cancer and Nutrition (EPIC): an observational study. *Lancet (London, England)*. **2003**;361(9368):1496-501.

Bingham S. A., Pignatelli B., Pollock J. R., Ellul A., Malaveille C., Gross G., Runswick S., Cummings J. H., O'Neill I. K. Does increased endogenous formation of N-nitroso compounds in the human colon explain the association between red meat and colon cancer? *Carcinogenesis*. **1996**;17(3):515-23.

Bonnett R., Charalambides A. A., Martin R. A., Sales K. D., Fitzsimmons B. W. Reactions of nitrous acid and nitric oxide with porphyrins and haems. Nitrosylhaems as nitrosating agents. *Journal of the Chemical Society, Chemical Communications*. **1975**(21):884-5.

Calmels S., Ohshima H., Bartsch H. Nitrosamine formation by denitrifying and non-denitrifying bacteria: implication of nitrite reductase and nitrate reductase in nitrosation catalysis. *Journal of general microbiology*. **1988**;134(1):221-6.

Calmels S., Ohshima H., Henry Y., Bartsch H. Characterization of bacterial cytochrome cd(1)-nitrite reductase as one enzyme responsible for catalysis of nitrosation of secondary amines. *Carcinogenesis*. **1996**;17(3):533-6.

Chan D. S., Lau R., Aune D., Vieira R., Greenwood D. C., Kampman E., Norat T. Red and processed meat and colorectal cancer incidence: meta-analysis of prospective studies. *PLoS One*. **2011**;6(6):e20456.

Cross A. J., Pollock J. R., Bingham S. A. Haem, not protein or inorganic iron, is responsible for endogenous intestinal N-nitrosation arising from red meat. *Cancer research*. **2003**;63(10):2358-60.

Ganhão R., Morcuende D., Estévez M. Protein oxidation in emulsified cooked burger patties with added fruit extracts: Influence on colour and texture deterioration during chill storage. *Meat Science*. **2010**;85(3):402-9.

Gottschalg E., Scott G. B., Burns P. A., Shuker D. E. Potassium diazoacetate-induced p53 mutations in vitro in relation to formation of O6-carboxymethyl- and O6-methyl-2'-deoxyguanosine DNA adducts: relevance for gastrointestinal cancer. *Carcinogenesis*. **2007**;28(2):356-62.

Hernández P., Zomeño L., Ariño B., Blasco A. Antioxidant, lipolytic and proteolytic enzyme activities in pork meat from different genotypes. *Meat Science*. **2004**;66(3):525-9.

Hogg N. Red meat and colon cancer: Heme proteins and nitrite in the gut. A commentary on “Diet-induced endogenous formation of nitroso compounds in the GI tract”. *Free Radical Biology and Medicine*. **2007**;43(7):1037-9.

Hughes R., Cross A. J., Pollock J. R. A., Bingham S. Dose-dependent effect of dietary meat on endogenous colonic N-nitrosation. *Carcinogenesis*. **2001**;22(1):199-202.

Hughes R., Magee E. A., Bingham S. Protein degradation in the large intestine: relevance to colorectal cancer. *Current issues in intestinal microbiology*. **2000**;1(2):51-8.

Hur S. J., Lee S. Y., Lee S. J. Effect of biopolymer encapsulation on the digestibility of lipid and cholesterol oxidation products in beef during in vitro human digestion. *Food chemistry*. **2014**;166:254-60.

Hur S. J., Lim B. O., Park G. B., Joo S. T. Effects of various fiber additions on lipid digestion during in vitro digestion of beef patties. *Journal of food science*. **2009**;74(9):C653-7.

Kobayashi J. Effect of diet and gut environment on the gastrointestinal formation of N-nitroso compounds: A review. *Nitric oxide : biology and chemistry*. **2018**;73:66-73.

Kuhnle G. G., Bingham S. A. Dietary meat, endogenous nitrosation and colorectal cancer. *Biochemical Society transactions*. **2007**;35(Pt 5):1355-7.

Kuhnle G. G., Story G. W., Reda T., Mani A. R., Moore K. P., Lunn J. C., Bingham S. A. Diet-induced endogenous formation of nitroso compounds in the GI tract. *Free radical biology & medicine*. **2007**;43(7):1040-7.

Le Leu R. K., Winter J. M., Christophersen C. T., Young G. P., Humphreys K. J., Hu Y., Gratz S. W., Miller R. B., Topping D. L., Bird A. R., Conlon M. A. Butyrylated starch intake can prevent red meat-induced O6-methyl-2-deoxyguanosine adducts in human rectal tissue: a randomised clinical trial. *The British journal of nutrition*. **2015**;114(2):220-30.

Lin S., Hsieh F., Huff H. E. Effects of lipids and processing conditions on lipid oxidation of extruded dry pet food during storage. *Animal Feed Science and Technology*. **1998**;71(3):283-94.

Mallett A. K., Rowland I. R., Bearne C. A. Influence of wheat bran on some reductive and hydrolytic activities of the rat cecal flora. *Nutrition and cancer*. **1986**;8(2):125-31.

Mallett A. K., Wise A., Rowland I. R. Effect of dietary cellulose on the metabolic activity of the rat caecal microflora. *Archives of toxicology*. **1983**;52(4):311-7.

Massey R. C., Key P. E., Mallett A. K., Rowland I. R. An investigation of the endogenous formation of apparent total N-nitroso compounds in conventional microflora and germ-free rats. *Food and Chemical Toxicology*. **1988**;26(7):595-600.

Mirvish S. S. Role of N-nitroso compounds (NOC) and N-nitrosation in etiology of gastric, esophageal, nasopharyngeal and bladder cancer and contribution to cancer of known exposures to NOC. *Cancer Letters*. **1995**;93(1):17-48.

Møller M. E., Dahl R., Bøckman O. C. A possible role of the dietary fibre product, wheat bran, as a nitrite scavenger. *Food and Chemical Toxicology*. **1988**;26(10):841-5.

Nair U., Bartsch H., Nair J. Lipid peroxidation-induced DNA damage in cancer-prone inflammatory diseases: A review of published adduct types and levels in humans. *Free Radical Biology and Medicine*. **2007**;43(8):1109-20.

Silvester K. R., Bingham S. A., Pollock J. R., Cummings J. H., O'Neill I. K. Effect of meat and resistant starch on fecal excretion of apparent N-nitroso compounds and ammonia from the human large bowel. *Nutrition and cancer*. **1997**;29(1):13-23.

Thogersen R., Castro-Mejia J. L., Sundekilde U. K., Hansen L. H., Hansen A. K., Nielsen D. S., Bertram H. C. Ingestion of an Inulin-Enriched Pork Sausage Product Positively Modulates the Gut Microbiome and Metabolome of Healthy Rats. *Molecular nutrition & food research*. **2018**:e1800608.

Toden S., Belobrajdic D. P., Bird A. R., Topping D. L., Conlon M. A. Effects of dietary beef and chicken with and without high amylose maize starch on blood malondialdehyde, interleukins, IGF-I, insulin, leptin, MMP-2, and TIMP-2 concentrations in rats. *Nutrition and cancer*. **2010**;62(4):454-65.

Toden S., Bird A. R., Topping D. L., Conlon M. A. High red meat diets induce greater numbers of colonic DNA double-strand breaks than white meat in rats: attenuation by high-amylose maize starch. *Carcinogenesis*. **2007**;28(11):2355-62.

Van Hecke T., Ho P. L., Goethals S., De Smet S. The potential of herbs and spices to reduce lipid oxidation during heating and gastrointestinal digestion of a beef product. *Food research international (Ottawa, Ont)*. **2017**;102:785-92.

Van Hecke T., Vanden Bussche J., Vanhaecke L., Vossen E., Van Camp J., De Smet S. Nitrite curing of chicken, pork, and beef inhibits oxidation but does not affect N-nitroso compound (NOC)-specific DNA adduct formation during in vitro digestion. *Journal of agricultural and food chemistry*. **2014**;62(8):1980-8.

Winter J., Nyskohus L., Young G. P., Hu Y., Conlon M. A., Bird A. R., Topping D. L., Le Leu R. K. Inhibition by resistant starch of red meat-induced promutagenic adducts in mouse colon. *Cancer prevention research (Philadelphia, Pa)*. **2011**;4(11):1920-8.

Yamauchi R., Goto Y., Kato K., Ueno Y. Prooxidant Effect of Dihydroxyacetone and Reducing Sugars on the Autoxidation of Methyl Linoleate in Emulsions. *Agricultural and Biological Chemistry*. **1984**;48(4):843-8.

Figure 1. Concentration of fecal nitroso compounds after 4 weeks of intervention in rats, mean ± SEM (Chow, n = 6; Sausage + inulin, n = 12; control sausage, n = 12). Different letters within each compound class indicate significant differences between control sausage and inulin sausage. ATNC, apparent total N-nitroso compounds; RSNO, nitrosothiols; FeNO, nitrosyl iron compounds; other, remaining unspecified nitroso compounds. Concentrations for each individual rat can be found in supplementary material (Table S3). For the chow diet group, all nitroso compounds analyzed were below the detection limit.

Figure 2. PCA scores plot of NMR metabolite profiles obtained for liver samples from rats fed inulin-enriched sausages (yellow), control sausages (red) or chow diet (blue) for 4 weeks.

Figure 3. OPLS-DA S-line plot of liver samples from rats fed either of the two sausage-based diets (n = 24) versus standard chow diet for 4 weeks (n = 6), $Q^2 = 0.79$.

$NaNO_2$ concentration (µM)	Control (AUC)	+ inulin (AUC)	p-value
50	240.33 ± 6.35	229.54 ± 1.21	0.28
40	179.56 ± 2.69	167.91 ± 5.18	0.12
30	88.52 ± 9.39	104.88 ± 9.42	0.29
20	60.86 ± 2.11	60.87 ± 0.58	1.00
10	20.47 ± 1.10	21.60 ± 0.75	0.44
5	8.25 ± 0.48	8.01 ± 1.27	0.87
2.5	3.58 ± 0.29	3.30 ± 0.55	0.67

Table 1. ATNC formation expressed as area under the curve (AUC) (mean ± SEM, n = 3) for incubation of bovine hemoglobin and varying amounts of sodium nitrite under acidic conditions with or without (control) the addition of inulin.

		Chow	Sausage + inulin	Control sausage	p-value
Liver	MDA (nmol/g liver)	419.70 ± 15.09	395.61 ± 9.52	399.09 ± 8.60	0.33
	GSH-Px (U/g)	51.3 ± 2.7^{a}	73.5 ± 1.7^{b}	77.6 ± 2.9^{b}	<0.001
	PCC (nmol DNPH/mg protein)	4.41 ± 0.19	4.28 ± 0.23	4.83 ± 0.33	0.31
Plasma	MDA (nmol/mL)	9.51 ± 0.61	9.83 ± 0.30	9.83 ± 0.21	0.81
	GSH-Px (U/mL)	1.96 ± 0.01	2.32 ± 0.15	2.41 ± 0.08	0.07
Diets	MDA (nmol/g diet)	65.5 ± 4.2^{a}	39.4 ± 0.35^{b}	32.0 ± 1.0^{b}	<0.001
	4-HNE (ng/g diet)	64.1 ± 7.5^{a}	4.2 ± 0.91^{b}	3.7 ± 0. ^{b}	<0.001
	HEX (ng/g diet)	508.9 ± 26.1^{a}	7.4 ± 0.2 ^{b}	8.4 ± 2.1^{b}	<0.001
	PCC (nmol DNPH/mg protein)	13.6 ± 0.5^{a}	9.5 ± 0.5^{c}	7.4 ± 0.2^{b}	<0.001
In vitro digest	MDA (nmol/g digest)	76.4 ± 4.0^{a}	25.8 ± 0.9^{b}	24.1 ± 0.2^{b}	<0.001
	4-HNE (ng/g digest)	20.4 ± 0.9^{a}	3.4 ± 0.3^{b}	4.2 ± 0.1^{b}	<0.001
	HEX (ng/g digest)	87.4 ± 1.0^{a}	6.4 ± 0.4^{b}	7.4 ± 0.5^{b}	<0.001
	PCC (nmol DNPH/mg protein)	13.7 ± 0.2^{a}	8.3 ± 0.2^{b}	8.0 ± 0.4^{b}	<0.001

Table 2. Determination of oxidation parameters of rat liver, plasma, experimental diets and in vitro digestion of experimental diets, mean ± SEM (For liver and plasma: chow, n = 6; Sausage + inulin, n = 12; control sausage, n = 12, except for MDA in plasma: chow, n = 4; Sausage + inulin, n = 10; control sausage, n = 10 and PCC in liver: chow, n = 6; Sausage + inulin, n = 12; control sausage, n = 10. For diets and in vitro digest, n = 3 for each diet group). MDA, malondialdehyde; GSH-Px, Glutathione peroxidase; 4-HNE, 4-hydroxy-2-neonenal; HEX, hexanal; PCC, protein carbonyl compounds.

***Declaration of Interest Statement**

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Figure 1
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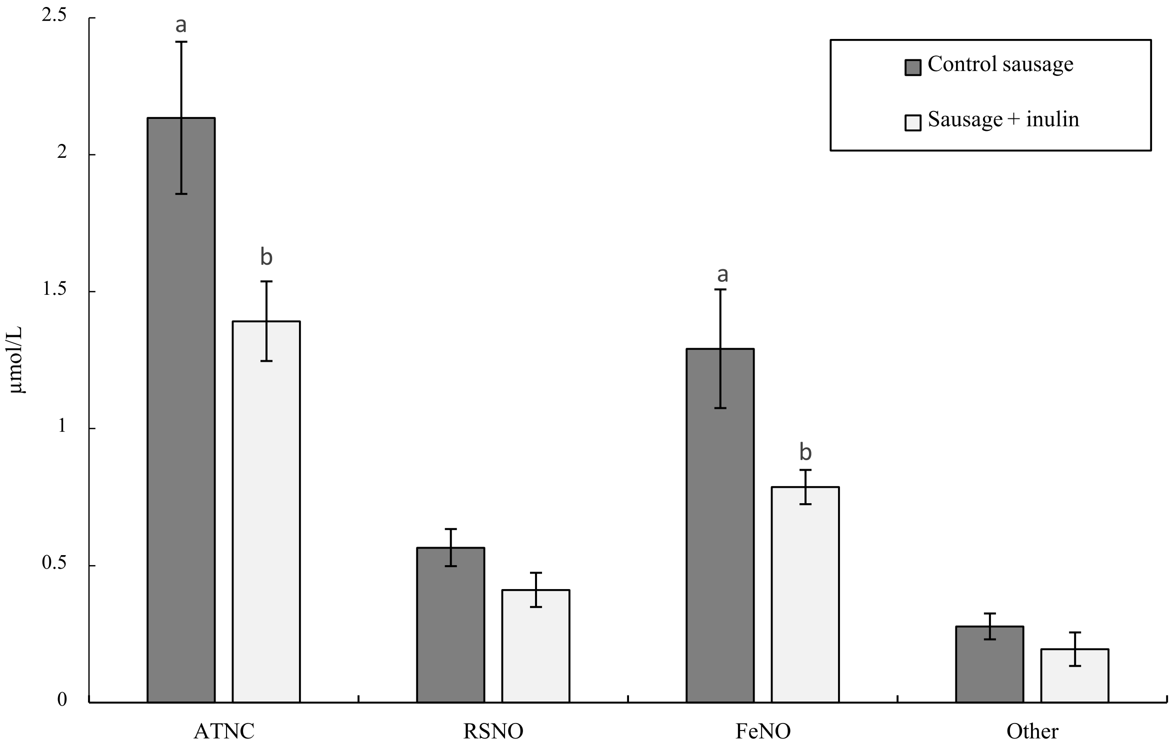

Figure 2

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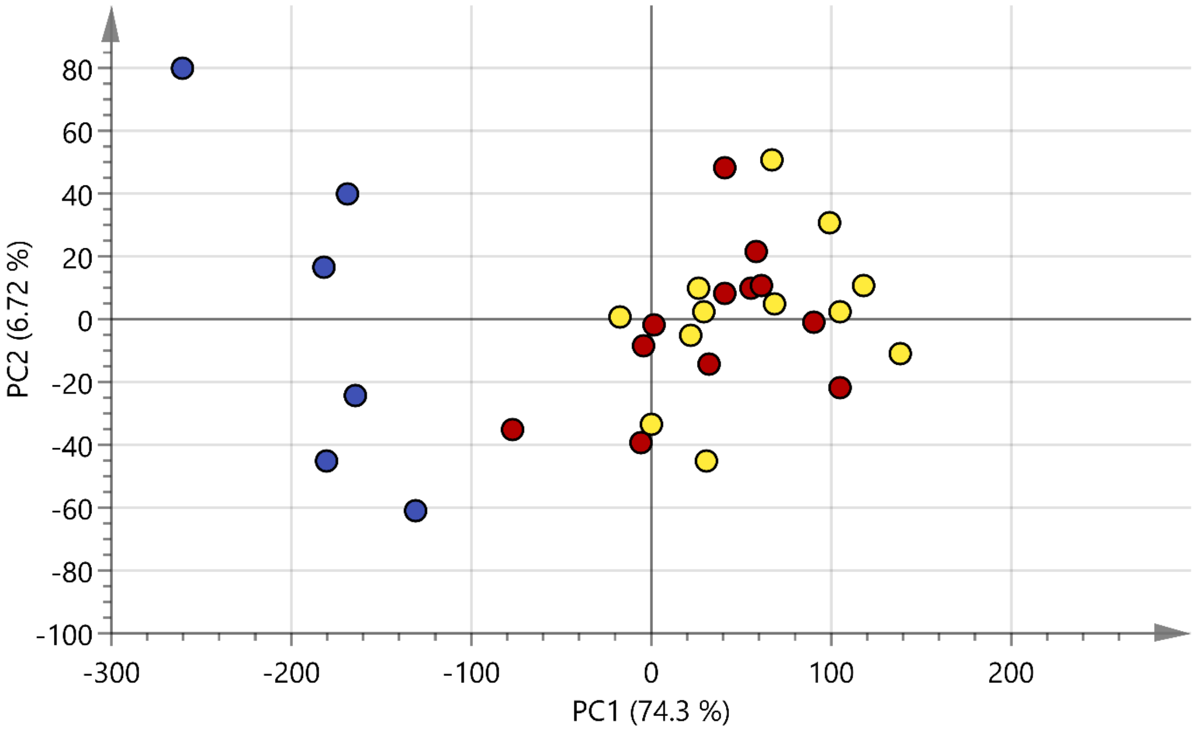

Figure 3
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Supplementary material

per 100 g	Control sausage	Inulin-enriched sausage	Chow
Energy (kcal)	245.0	259.0	318.9
Fat (g)	19.00	20.00	2.80
- Saturated	7.20	7.30	0.47
- Monounsaturated	8.30	8.40	0.63
- Polyunsaturated	2.60	3.10	1.69
Carbohydrate (g)	6.40	7.80	40.83
Protein (g)	12.00	12.00	19.19
Dietary fiber* (g)	0.00	5.60	6.05
NaCl (g)	2.50	2.50	0.54

Table S1. Nutritional content of diets.

**Calculated values*

Ingredient (% w/w)	Inulin-enriched sausage	Control sausage
Inulin	6.0	-
Salt (with 0.3nitrite)	2.0	2.0
Spices	2.0	2.0
AIN76 mineral mix: TD79055	2.0	2.0
AIN76 vitamin mix: CA40077	0.6	0.6
Choline bitartrate: CA30190	0.12	0.12

Sunflower oil	2.0	2.0

Table S2. Ingredients added to emulsion of minced pork meat and pork back fat during manufacturing of the two sausage-based diets; pork sausages enriched with inulin and control pork sausages.

	Concentration [umol/L]			
	ATNC	**RSNO**	**FeNO**	**Other**
Rat ID	Control sausage			
24	1.54	0.66	0.69	0.20
6	1.21	0.33	0.89	0.00
12	1.64	0.47	0.77	0.40
4	1.78	0.50	0.94	0.34
10	1.47	0.41	1.06	0.00
2	1.45	0.32	0.78	0.34
17	2.18	0.73	1.30	0.15
25	3.88	1.06	2.61	0.21
3	2.50	0.85	1.23	0.43
29	2.11	0.44	1.32	0.35
11	4.20	0.70	3.03	0.47
8	1.64	0.32	0.88	0.44
	Sausage + inulin			
26	1.18	0.10	0.92	0.17
9	0.96	0.24	0.71	0.00
30	1.38	0.50	0.88	0.00
16	1.84	0.69	0.86	0.29
23	0.70	0.14	0.56	0.00
7	2.38	0.76	1.05	0.57
18	0.95	0.24	0.27	0.44
14	1.01	0.25	0.72	0.04
28	1.27	0.51	0.73	0.03
27	1.65	0.47	0.80	0.38
19	1.30	0.42	0.88	0.00
5	2.08	0.61	1.05	0.41

Table S3. Concentration of fecal nitroso compounds after 4 weeks of intervention in rats. ATNC, apparent total N-nitroso compounds; RSNO, nitrosothiols; FeNO, nitrosyl iron compounds; other, remaining unspecified nitroso compounds. For the chow diet group, all nitroso compounds analyzed were below the detection limit.

	Chow	Sausage + inulin	Control sausage	p-value
MDA **(nmol/g DM)**	73.9 ± 4.7[a]	91.0 ± 0.8[b]	76.5 ± 2.4[a]	0.016
4-HNE **(ng/g DM)**	72.3 ± 8.4[a]	9.7 ± 2.1[b]	8.8 ± 1.9[b]	<0.001
HEX **(ng/g DM)**	573.7 ± 29.5[a]	17.0 ± 0.6[b]	20.1 ± 4.9[b]	<0.001

Table S4. Lipid oxidation of experimental diets expressed per gram dry matter, mean ± SEM (n = 3 for each diet group). MDA, malondialdehyde; 4-HNE, 4-hydroxy-2-nonenal; HEX, hexanal; DM, dry matter.

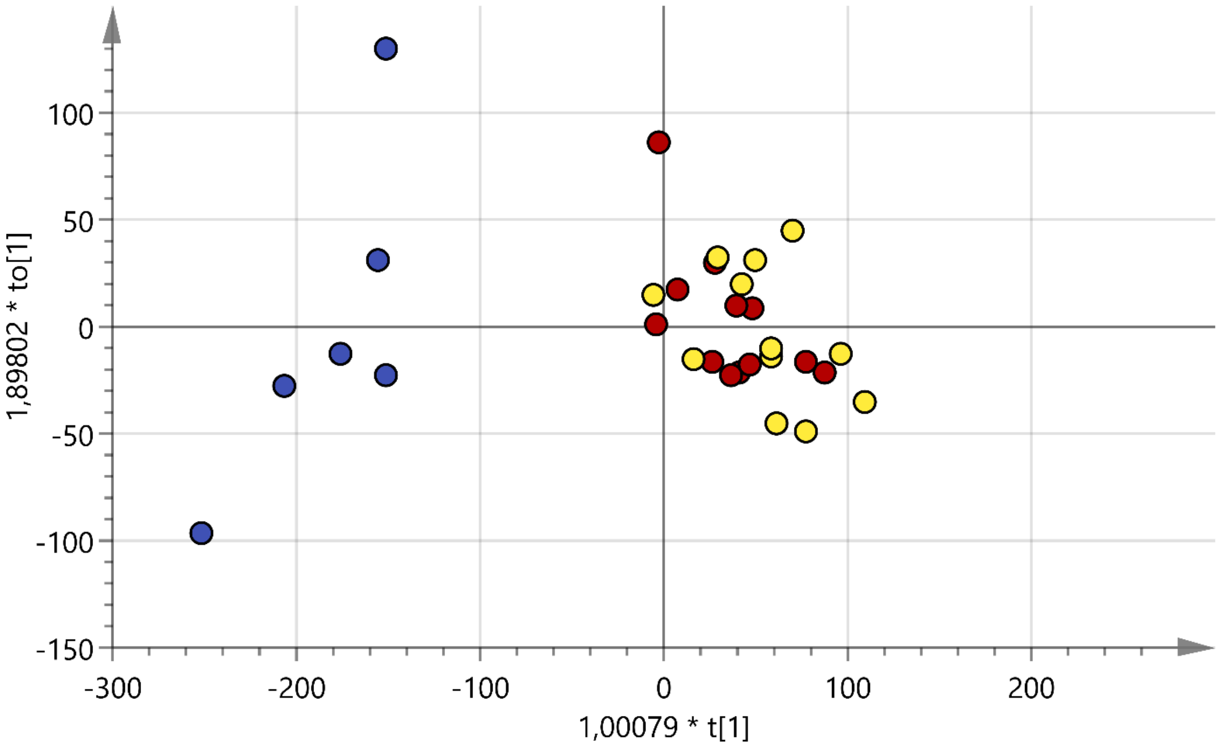

Figure S1. OPLS-DA scores plot of metabolite profiles obtained for liver samples from rats fed standard chow diet (blue), inulin-enriched sausage (yellow) or control sausage (red), Q^2 = 0.79.